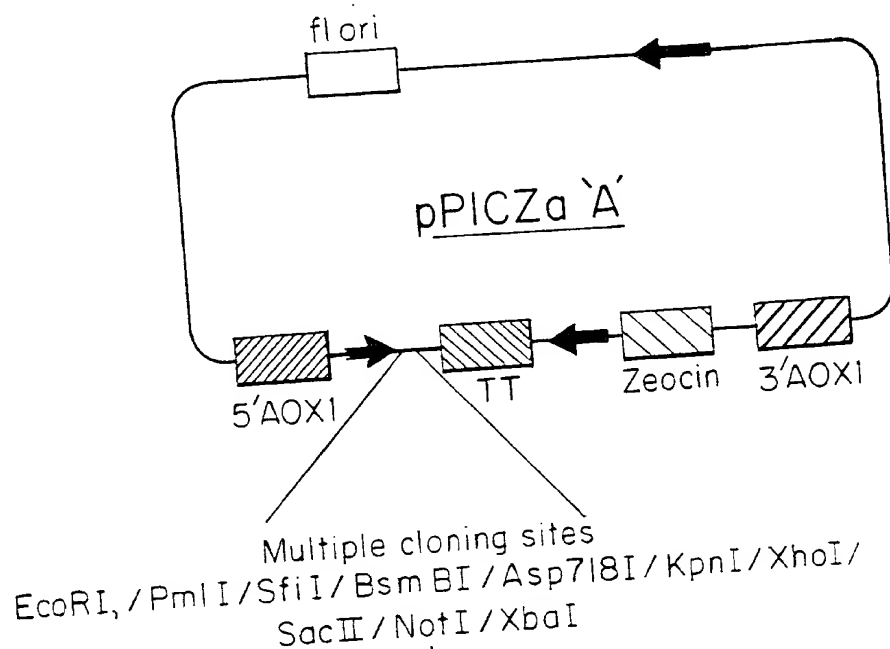


Fig. 1



Digest the vector with EcoRI restriction enzyme

Denature and anneal two complimentary oligo which would incorporate His.Tag motif and NdeI, NheI restriction sites (His. Tag region would be helpful to simplify purification and the presence of NdeI and NheI restriction sites facilitate shuttling of PCR from prokaryotic expression system to yeast expression without going through PCR amplification process)

5' AAT TCC ATC ACC ATC ACC ATC ACC ATA TGG CTA GCA 3'  
5' AAT TTG CTA GCC ATA TGG TGA TGG TGA TGG 3'

Modified Pichia expression vector (pPICZ. His. A)

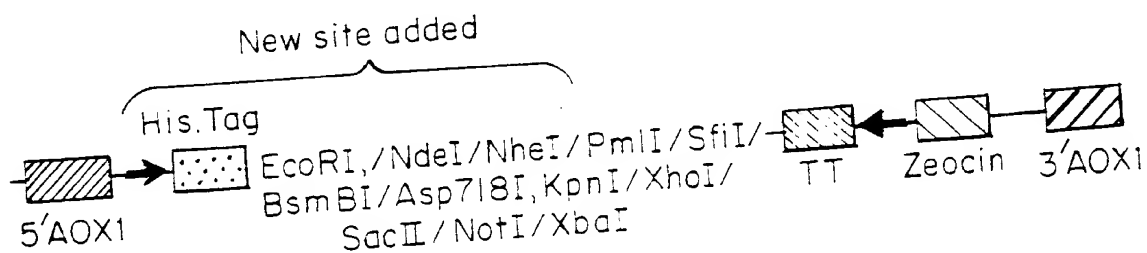


Fig. 2

# Flow Chart: Cloning of Endostatin (mouse) into Pichia Expression System

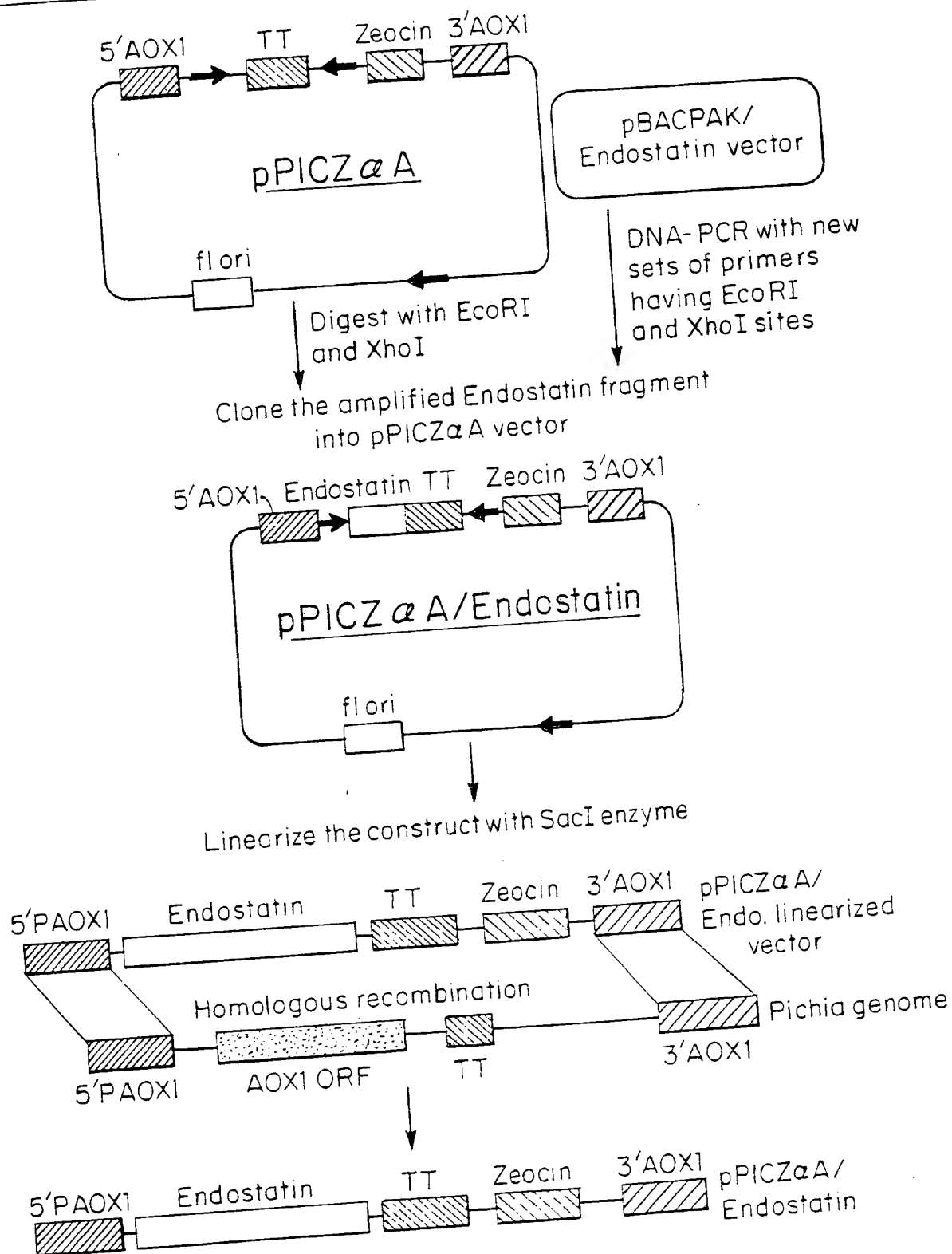


Fig. 3

endo sequence from Collagen XVIII.

Sequence Range: 1-555

Nucleotide 1 = Start for Endostatin and fragments EM1 and EM2.

EM1 fragment ends at nucleotide 525, EM2 fragment ends at nucleotide 501.

5	10	15	20	25	30	35	40	45							
CAT	ACT	CAT	CAG	GAC	TTT	CAG	CCA	GTG	CTC	CAG	CTG	GTG	GCA	CTG	AAC
GTA	TGA	GTA	GTC	CTG	AAA	GTC	GGT	CAC	GAG	GTG	GAC	CAC	CGT	GAC	TTG
50	55	60	65	70	75	80	85	90	95						
ACC	CCC	CTG	TCT	GGA	GGC	ATG	CGT	GGT	ATC	CGT	GGA	GCA	GAT	TTC	CAG
TGG	GGG	GAC	AGA	CCT	CCG	TAC	GCA	CCA	TAG	GCA	CCT	CGT	CTA	AAG	GTC
100	105	110	115	120	125	130	135	140							
TGC	TTC	CAG	CAA	GCC	CGA	GCC	GTG	GGG	CTG	TCG	GGC	ACC	TTC	CGG	GCT
ACG	AAG	GTC	GTT	CGG	GCT	CGG	CAC	CCC	GAC	AGC	CCG	TGG	AAG	GCC	CGA
145	150	155	160	165	170	175	180	185	190						
TTC	CTG	TCC	TCT	AGG	CTG	CAG	GAT	CTC	TAT	AGC	ATC	GTG	CGC	CGT	GCT
AAG	GAC	AGG	AGA	TCC	GAC	GTC	CTA	GAG	ATA	TCG	TAG	CAC	GCG	GCA	CGA
195	200	205	210	215	220	225	230	235	240						
GAC	CGG	GGG	TCT	GTG	CCC	ATC	GTC	AAC	CTG	AAG	GAC	GAG	GTG	CTA	TCT
CTG	GCC	CCC	AGA	CAC	GGG	TAG	CAG	TTG	GAC	TTC	CTG	CTC	CAC	GAT	AGA
245	250	255	260	265	270	275	280	285							
CCC	AGC	TGG	GAC	TCC	CTG	TTT	TCT	GGC	TCC	CAG	GGT	CAA	CTG	CAA	CCC
GGG	TCG	ACC	CTG	AGG	GAC	AAA	AGA	CCG	AGG	GTC	CCA	GTT	GAC	GTT	GGG
290	295	300	305	310	315	320	325	330	335						
GGG	GCC	CGC	ATC	TTT	TCT	TTT	GAC	GGC	AGA	GAT	GTC	CTG	AGA	CAC	CCA
CCC	CGG	GCG	TAG	AAA	AGA	AAA	CTG	CCG	TCT	CTA	CAG	GAC	TCT	GTG	GGT
340	345	350	355	360	365	370	375	380							
GCC	TGG	CCG	CAG	AAG	AGC	GTA	TGG	CAC	GGC	TCG	GAC	CCC	AGT	GGG	CGG
CGG	ACC	GGC	GTC	TTC	TCG	CAT	ACC	GTG	CCG	AGC	CTG	GGG	TCA	CCC	GCC
385	390	395	400	405	410	415	420	425	430						
AGG	CTG	ATG	GAG	AGT	TAC	TGT	GAG	ACA	TGG	CGA	ACT	GAA	ACT	ACT	GGG
TCC	GAC	TAC	CTC	TCA	ATG	ACA	CTC	TGT	ACC	GCT	TGA	CTT	TGA	TGA	CCC
435	440	445	450	455	460	465	470	475	480						
GCT	ACA	GGT	CAG	GCC	TCC	TCC	CTG	CTG	TCA	GGC	AGG	CTC	CTG	GAA	CAG
CGA	TGT	CCA	GTC	CGG	AGG	AGG	GAC	GAC	AGT	CCG	TCC	GAG	GAC	CTT	GTC
485	490	495	500	505	510	515	520	525							
AAA	GCT	GCG	AGC	TGC	CAC	AAC	AGC	TAC	ATC	GTC	CTG	TGC	ATT	GAG	AAT
TTT	CGA	CGC	TCG	ACG	GTG	TTG	TCG	ATG	TAG	CAG	GAC	ACG	TAA	CTC	TTA
530	535	540	545	550	555										
AGC	TTC	ATG	ACC	TCT	TTC	TCC	AAA	TAG							
TCG	AAG	TAC	TGG	AGA	AAG	AGG	TTT	ATC							

# ENDOSTATIN

ENDOSTATIN				
Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
MOUSE	ENDOSTATIN			
pET17b/ his.mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT'-3' (up) (SEQ ID NO:4)	NdeI & XhoI	Prokaryotic expression, pET ( <i>E. coli</i> his.endo)	MGHHHHHHHHHHSSGHHDDDDKH M-mendo (SEQ ID NO:14)
	5' AAC TCG AGC TAT TTG GAG AAA- GAG GT'-3' (down) (SEQ ID NO:5)			
	pET28a/ mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT'-3' (up) (SEQ ID NO:4)	NdeI & NcoI	
5'-AAG CGG CCG CCT ATT TGG AGA- AAG AGG TT'-3' (down) (SEQ ID NO:6)				
pET28a/ EM-1		5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		Prokaryotic expression, pET ( <i>E. coli</i> EM1)
	5' TTA GCG GCC GCC TAC TCA ATG- CAC AGG ACG ATG TA-3' (down) (SEQ ID NO:8)		Prokaryotic expression, pET ( <i>E. coli</i> EM2)	
	pET28a/ EM-2	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		
5' TTA GCG GCC GCC TAG TTG TGG- CAG CTC GCA GCT TTC TG-3' (down) (SEQ ID NO:9)				

Fig. 5A

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZ $\alpha$ A/ mendo  ( <i>yeast mus endo</i> )	5' GGG AAT TCC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:10)	EcoRI & NotI	Eukaryotic expression, yeast/pPICZ $\alpha$ A  ( <i>yeast mouse endostatin</i> )	EF-mendo (SEQ ID NO:16)
	5' AAG CCG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)			
	pPICZ $\alpha$ A/ His.mendo	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11)	EcoRI & NotI	Eukaryotic expression, yeast/pPICZ $\alpha$ A  ( <i>yeast mouse his.endostatin</i> )
	5' AAG CCG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)			
HUMAN				
pPICZ $\alpha$ A/ Hendo	5' TTT GAA TTC GCC CAC AGC CAC- CGC GAC TTC CAG CCG GTG CTC- CAC-3' (up) (SEQ ID NO:12)	EcoRI & NotI	Eukaryotic expression, yeast/pPICZ $\alpha$ A  ( <i>yeast human endostatin</i> )	EF-hendo (SEQ ID NO:18)
	5' AAA AGC GGC CGC CTA CTT GGA- GGC AGT CAT GAA GCT GTT CTC- AAT-3' (down) (SEQ ID NO:13)			

Fig. 5B

FIG.6A

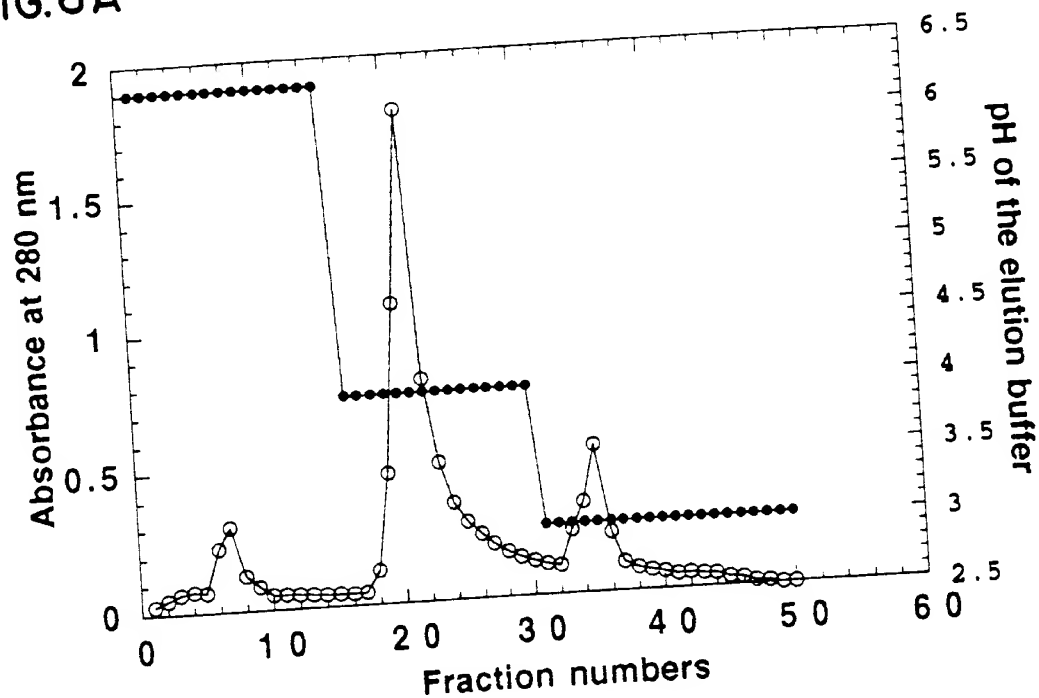


FIG.6B

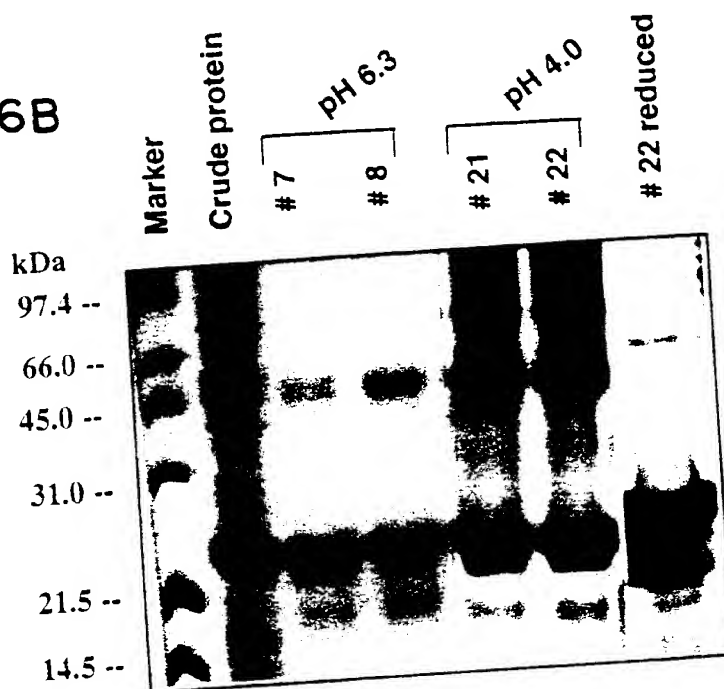


FIG.7A

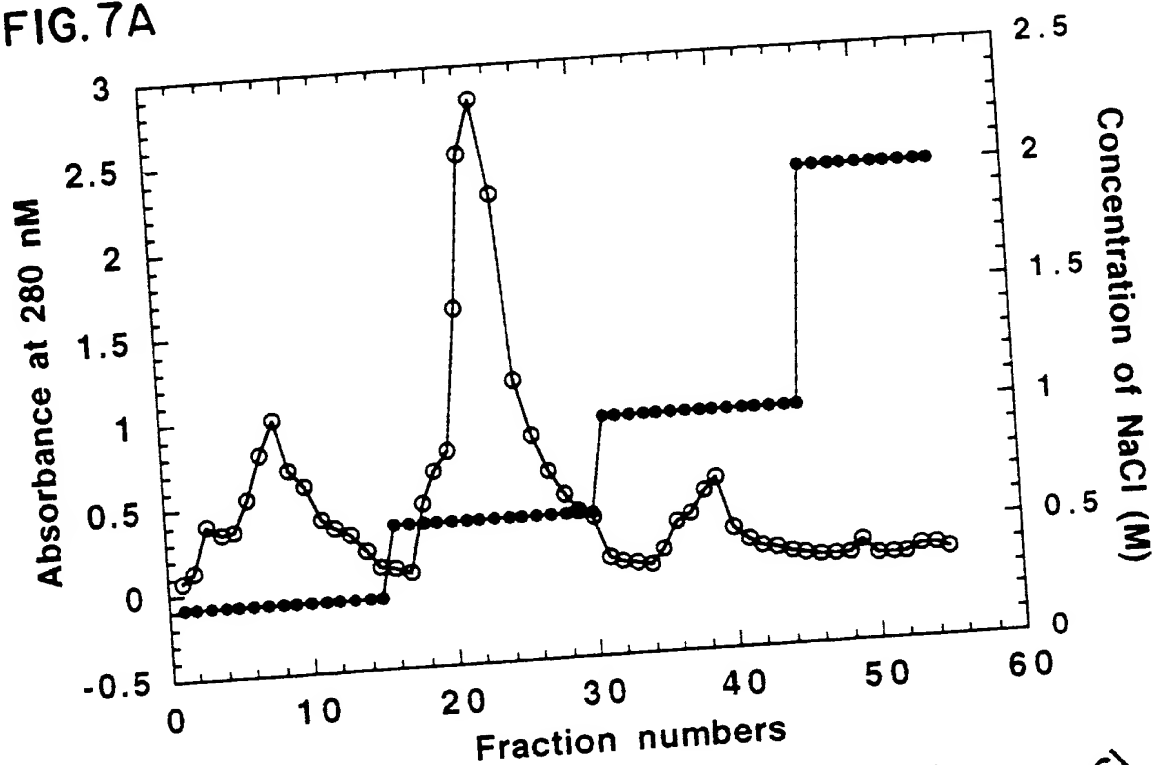


FIG.7B

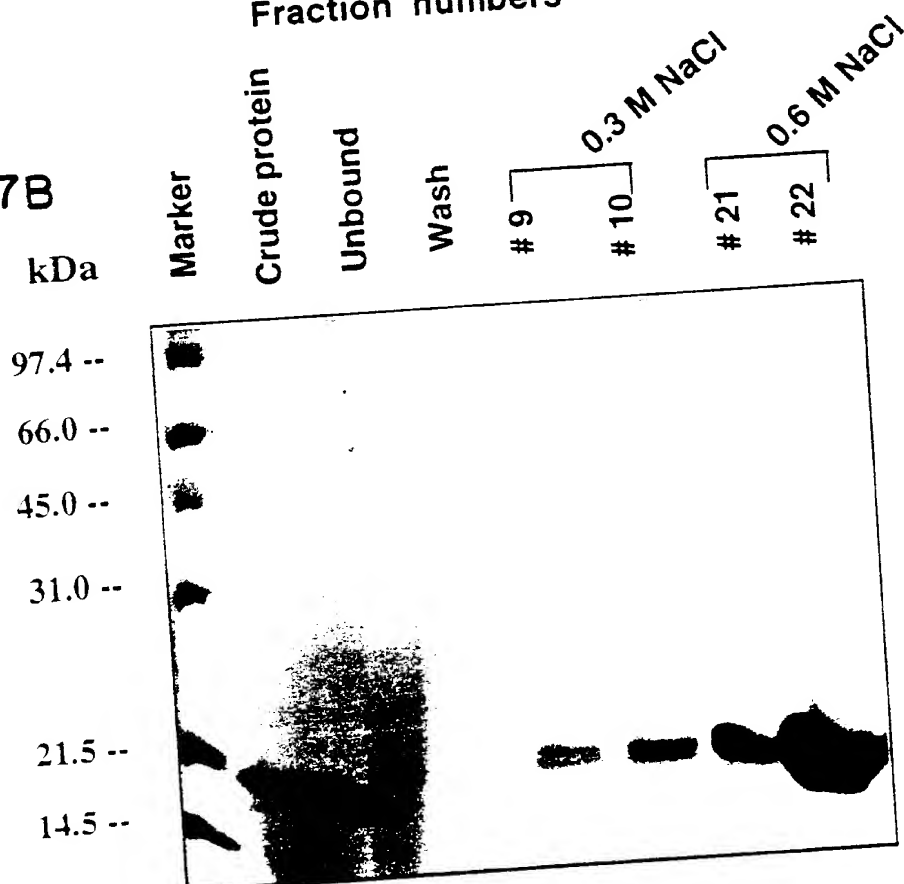




FIG.8A

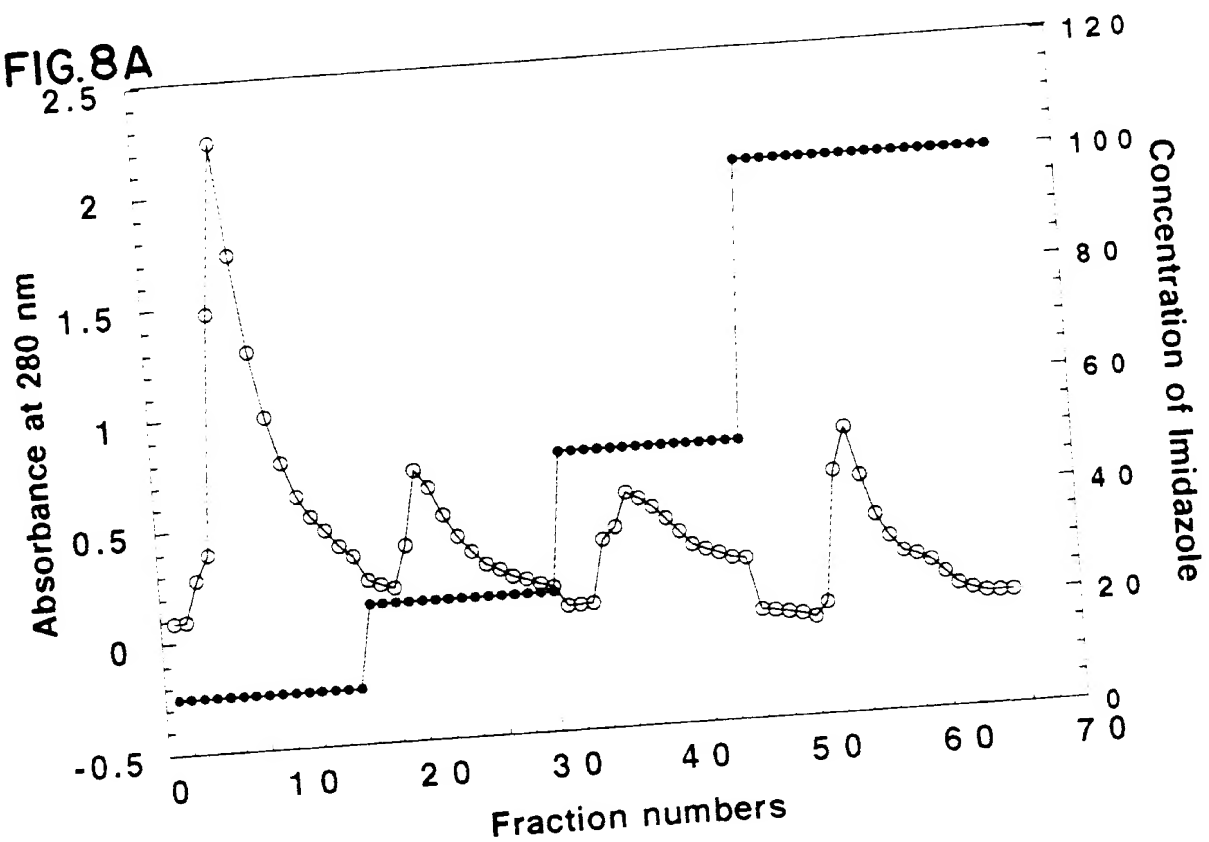
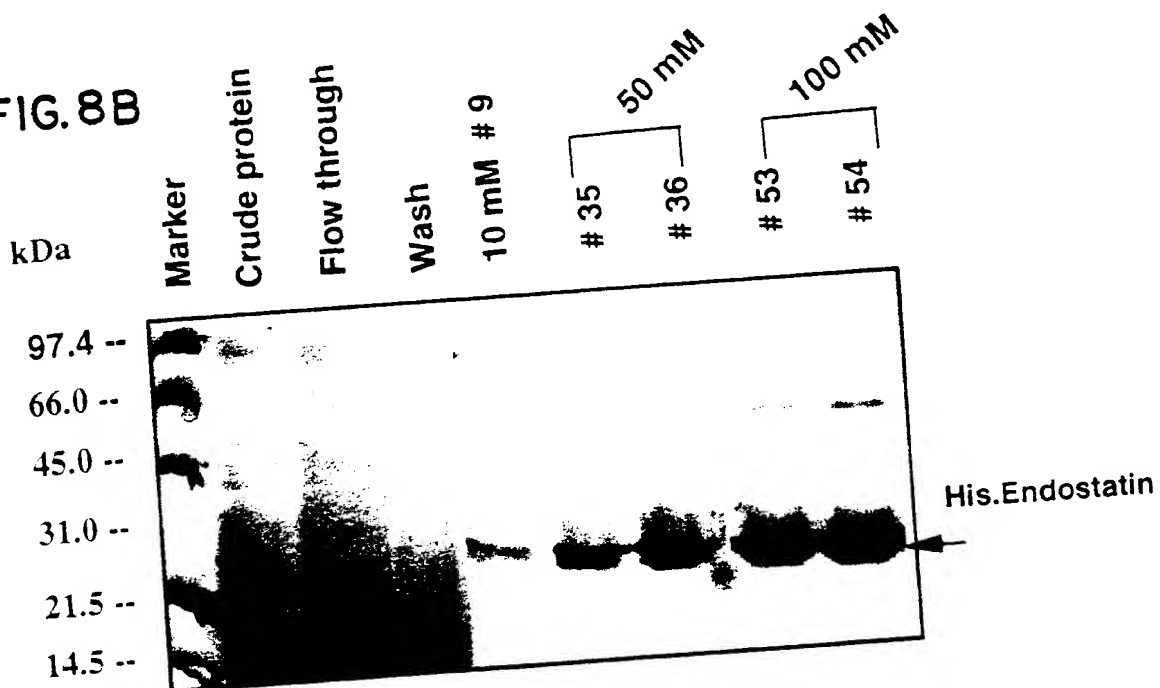


FIG.8B



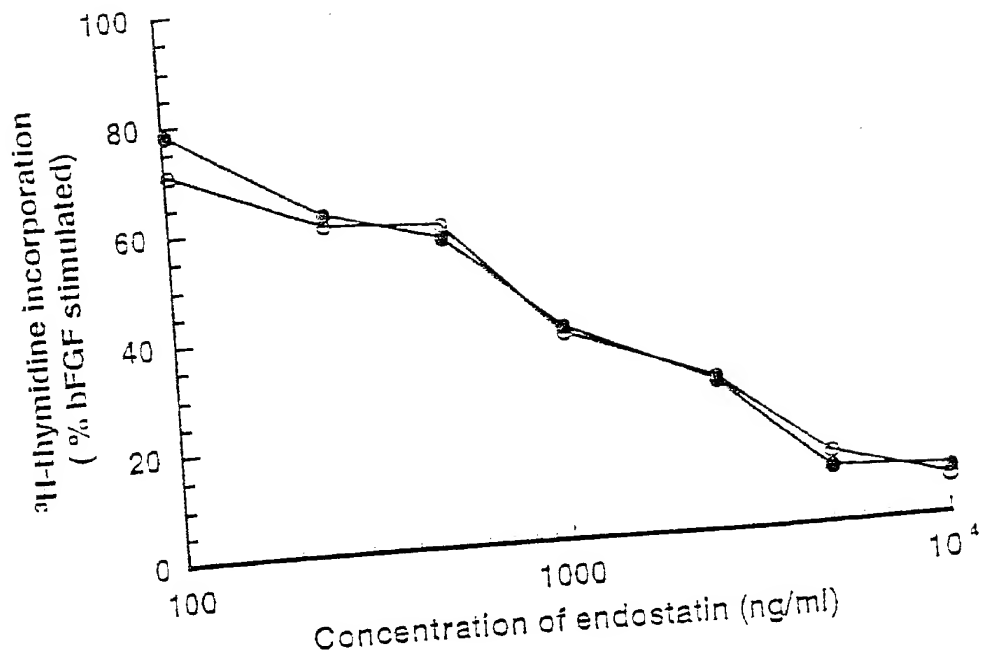


Fig. 9

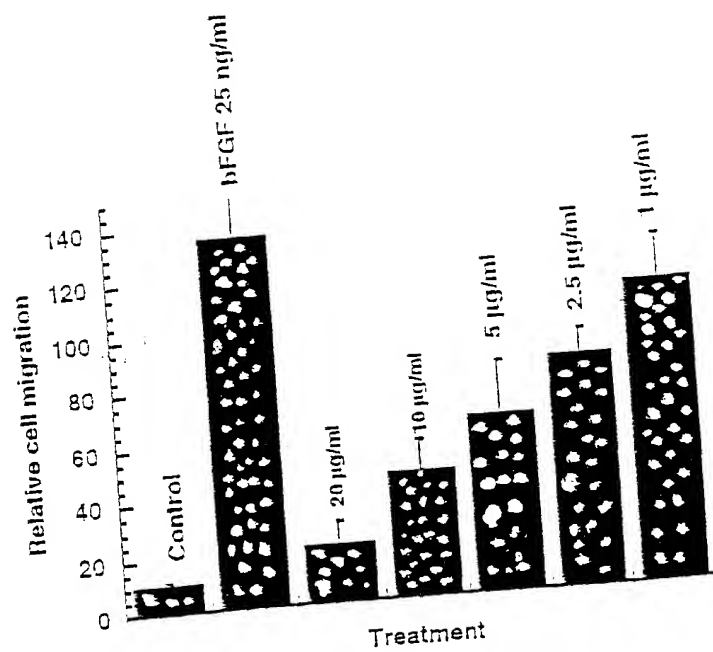


Fig. 10

Fig. 11A

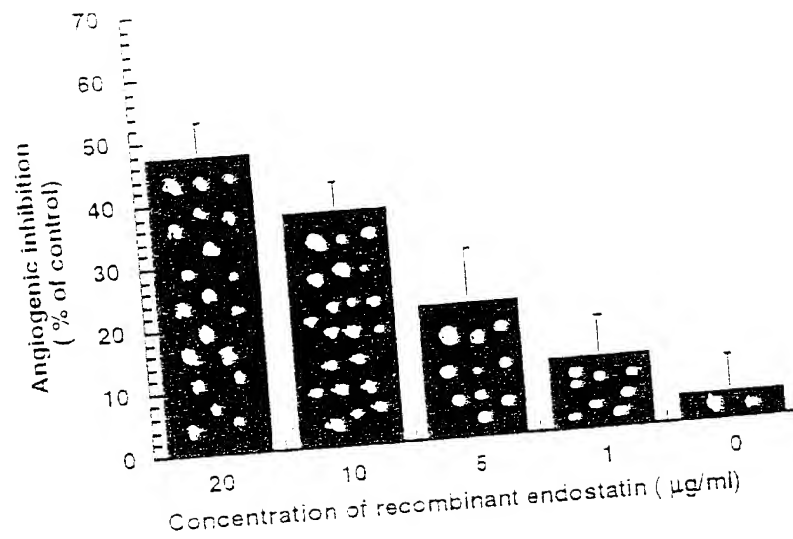
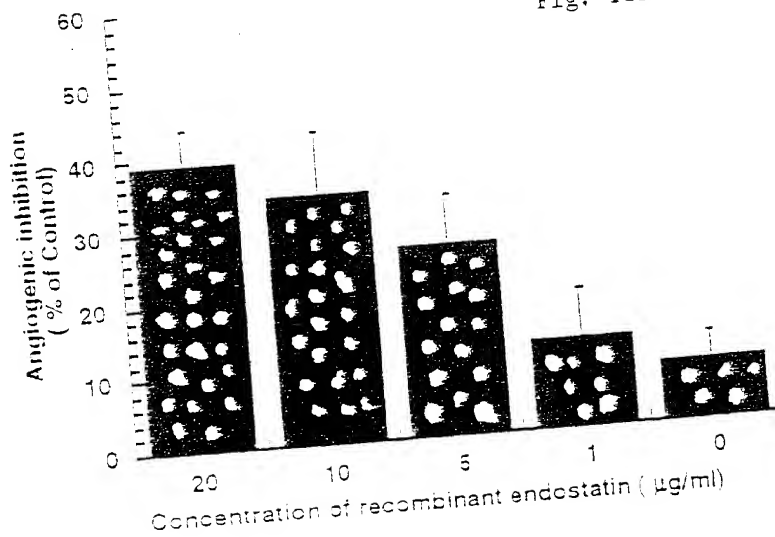


Fig. 11B.



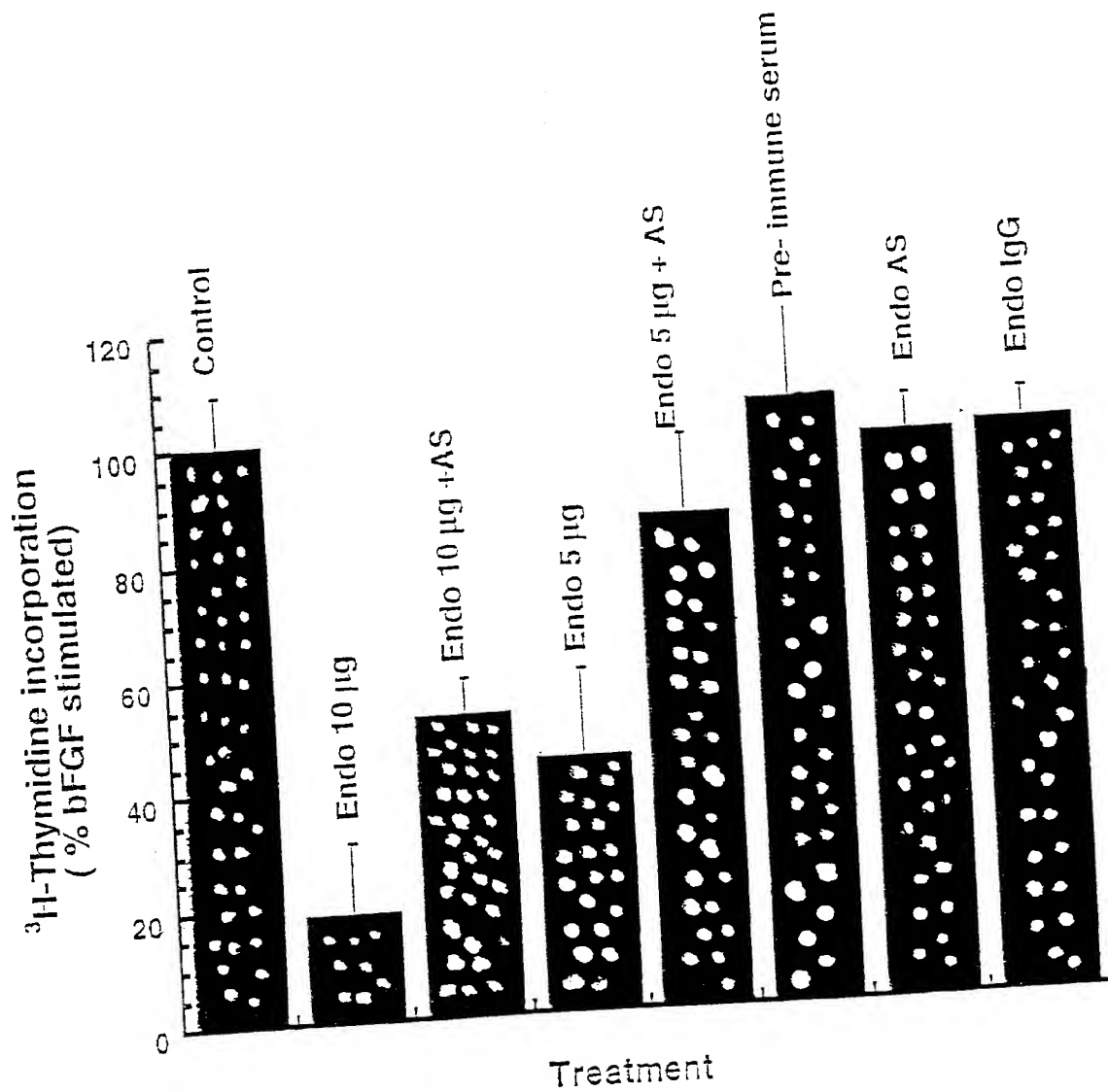


Fig. 12

Fig. 13A

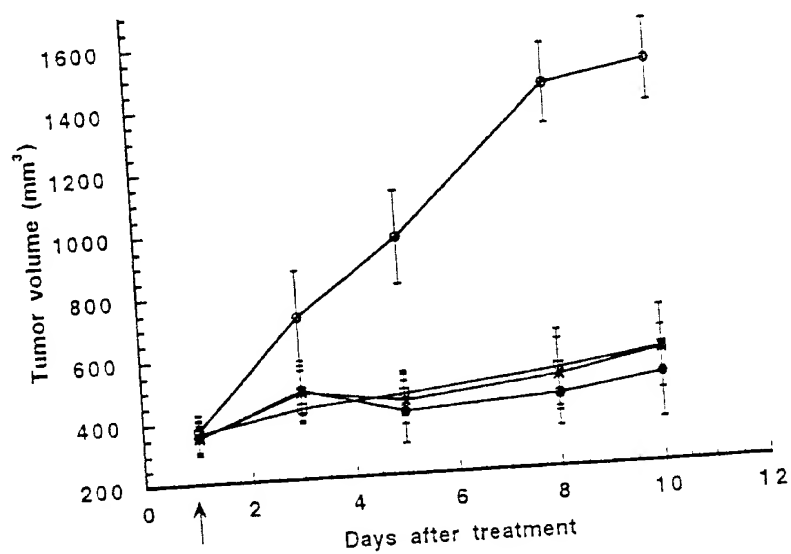


Fig. 13B

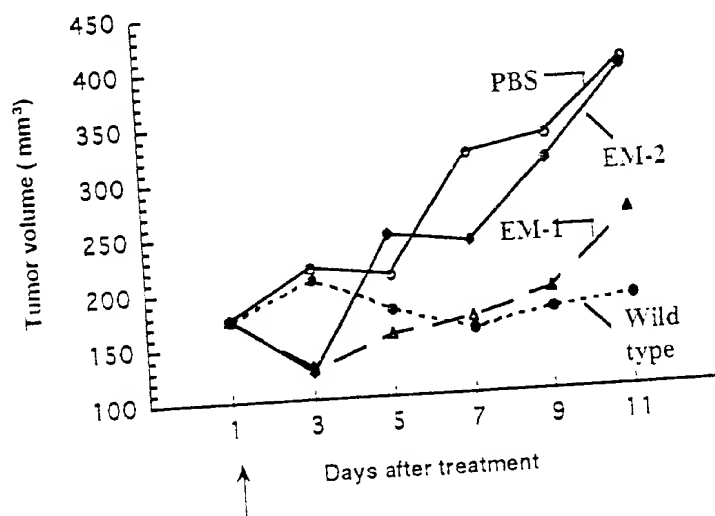


FIG.14A

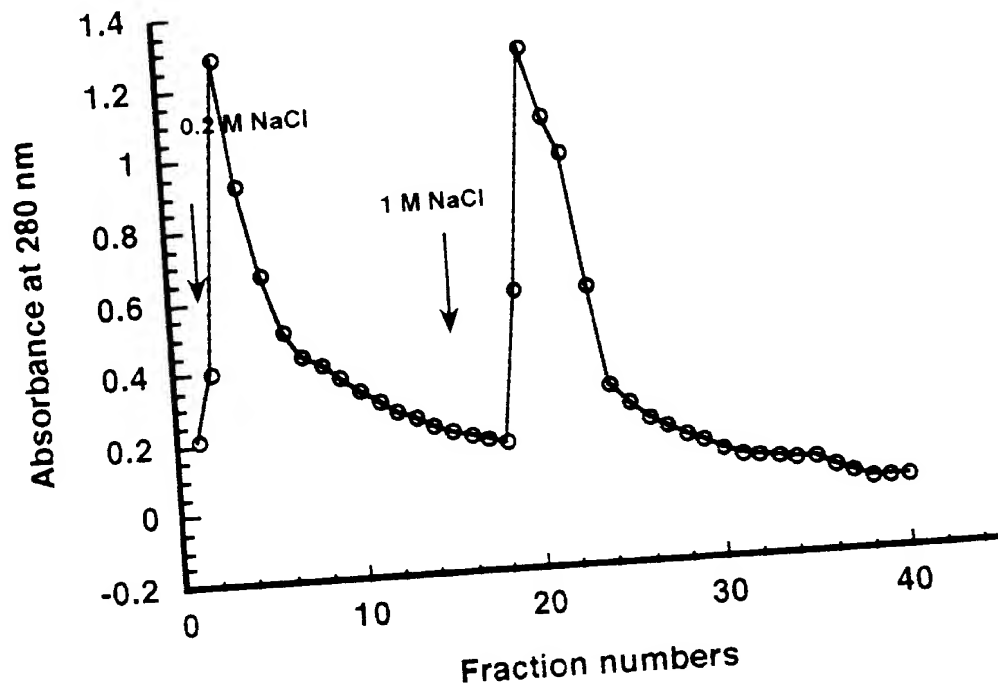


FIG.14B

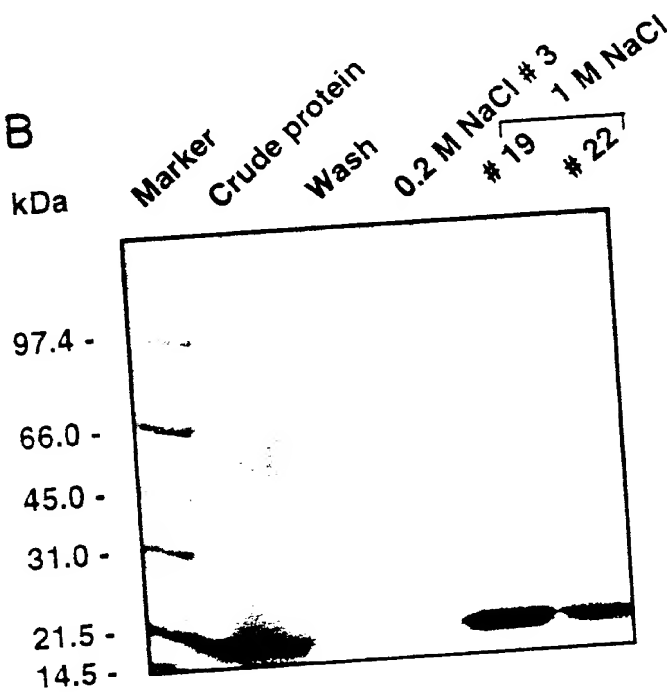


Fig. 15A

HUVE

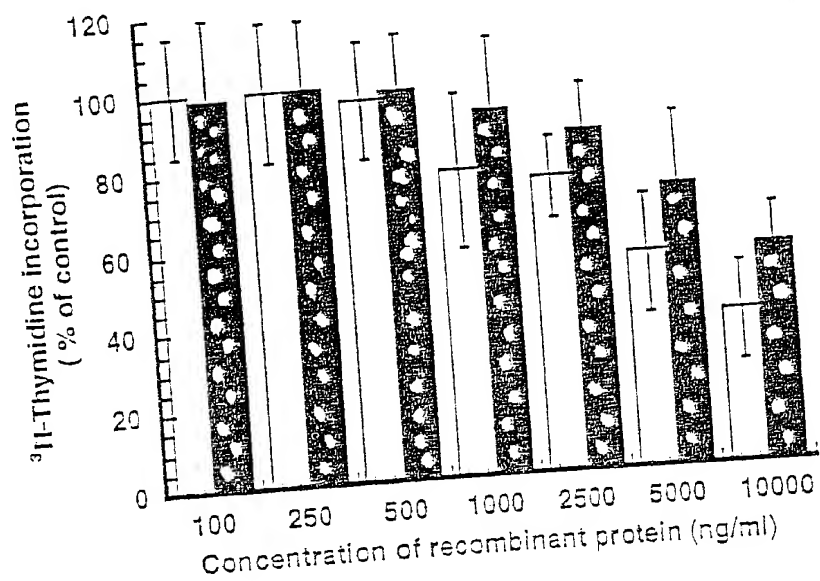


Fig. 15B

HMEV-L

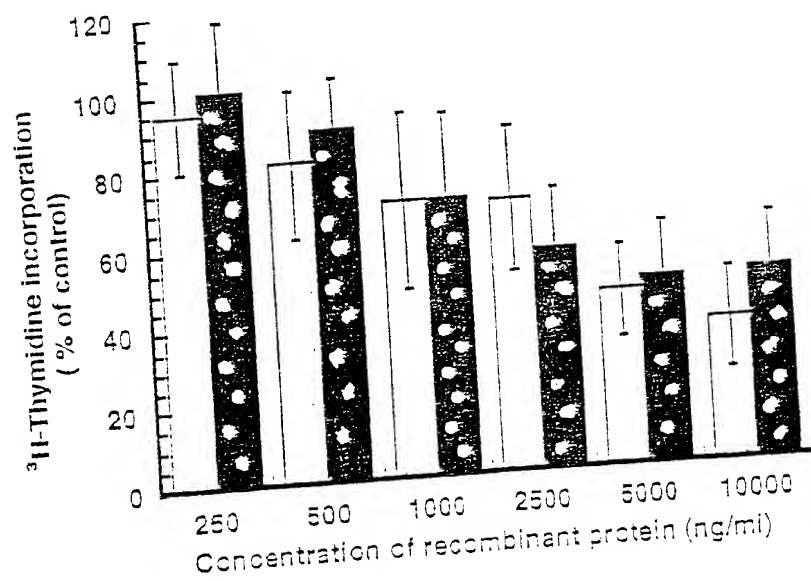




Fig. 16A

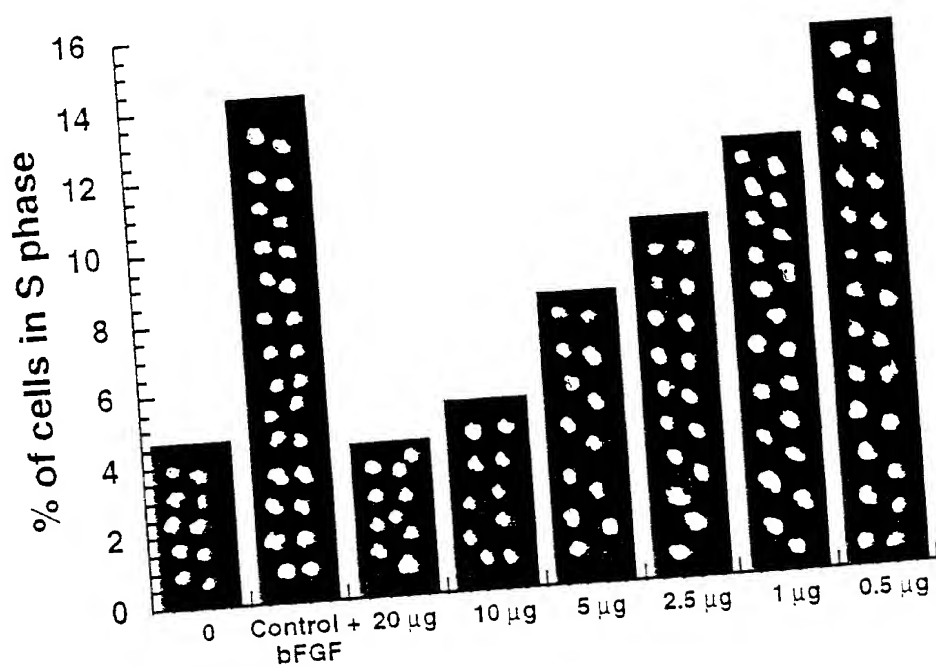


Fig. 16B

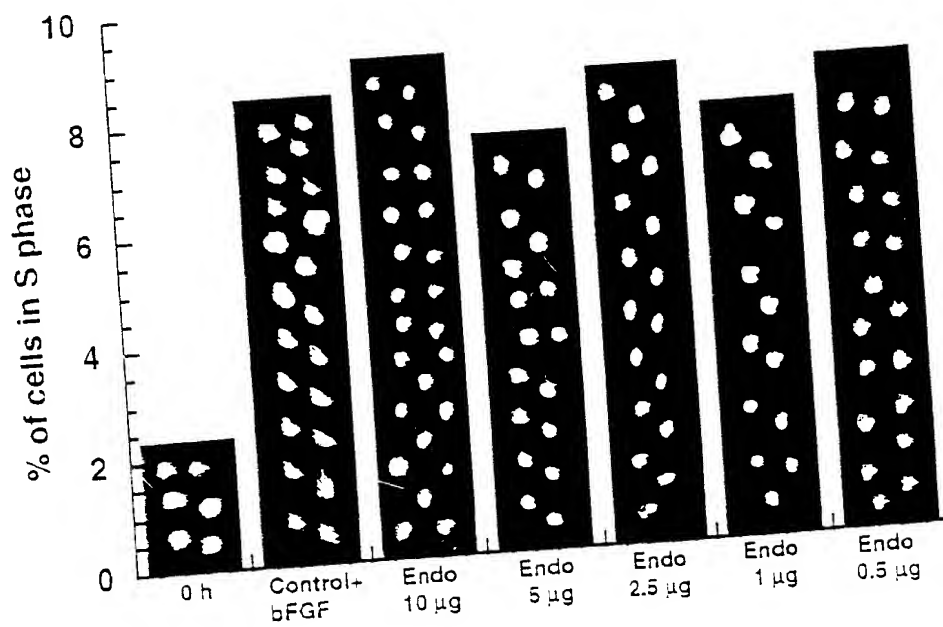


Fig. 16C

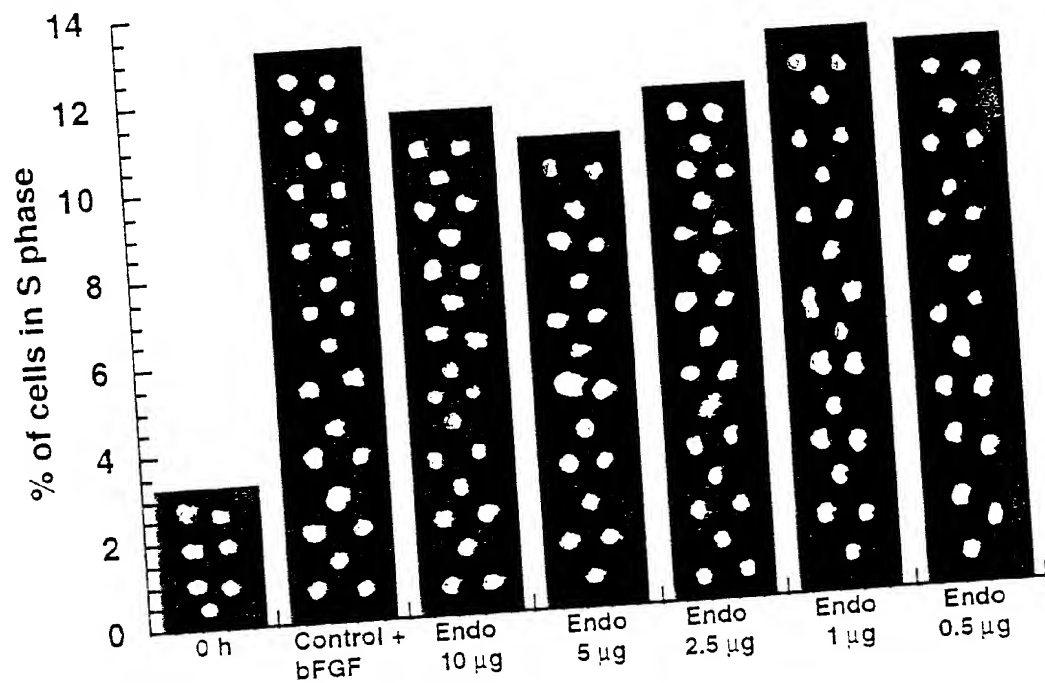
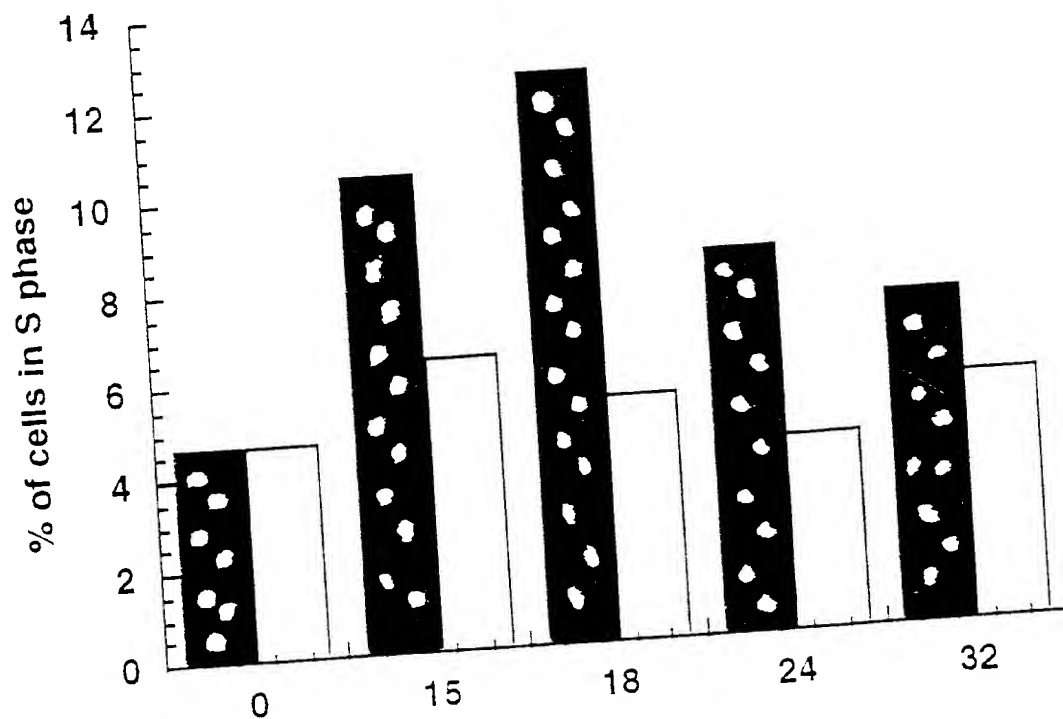


Fig. 16D



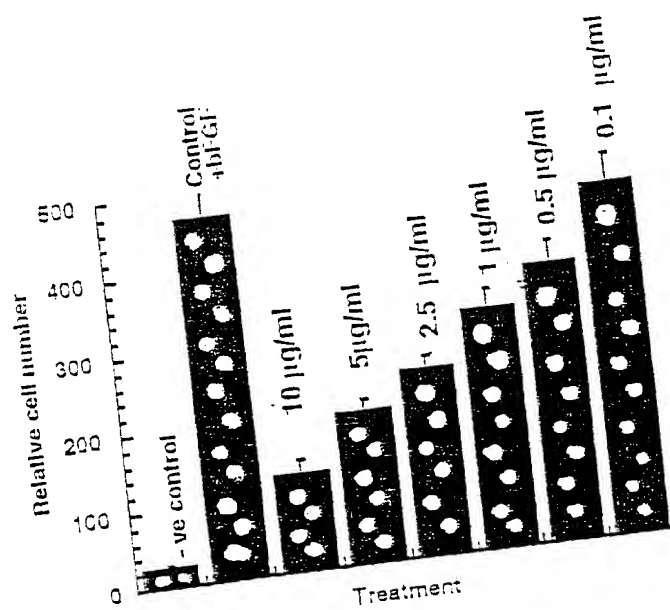
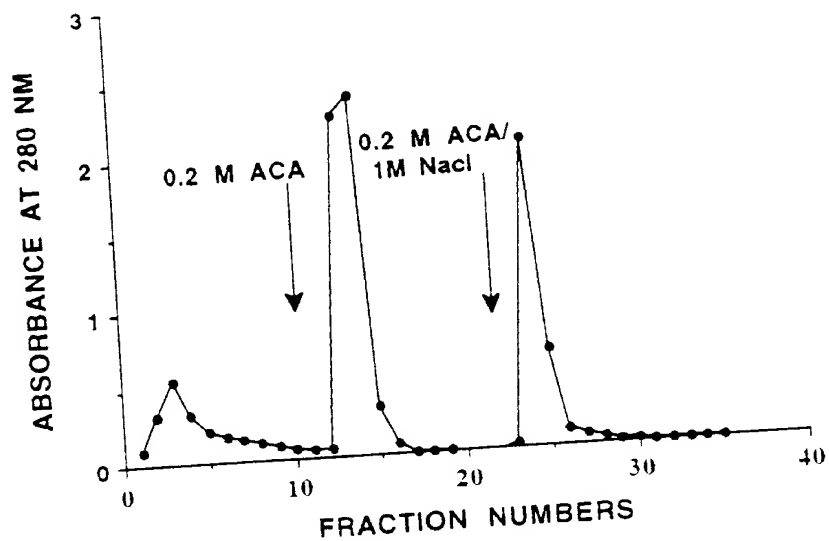


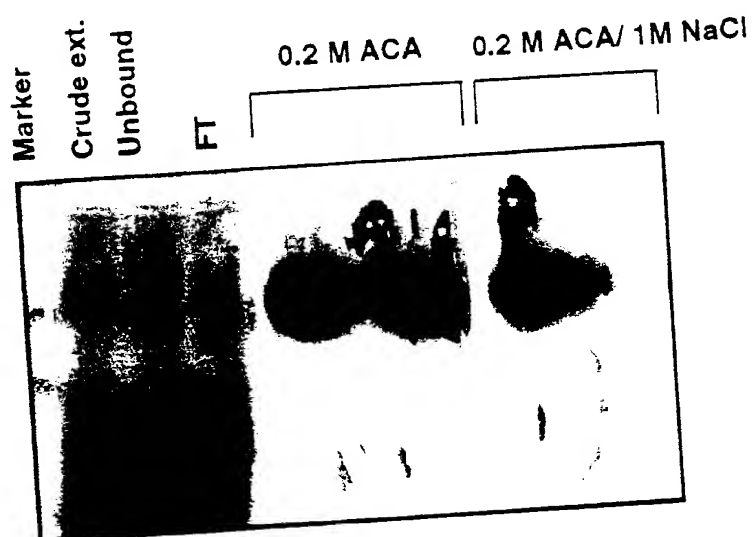
Fig. 17

**FIG.18A**

Purification of Angiostatin(mouse): Lysine Sepharose 4B column



**FIG.18B**



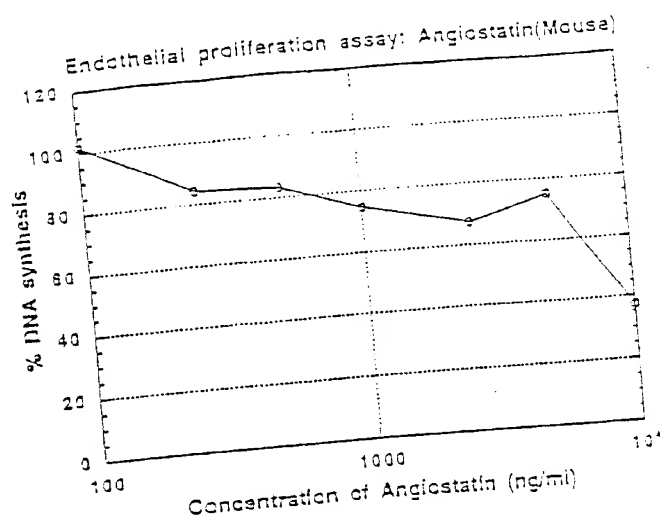


Fig. 19

Effect of Recombinant Endostatin (mouse) on tumor growth  
(Renal cancer cell line 786-0)

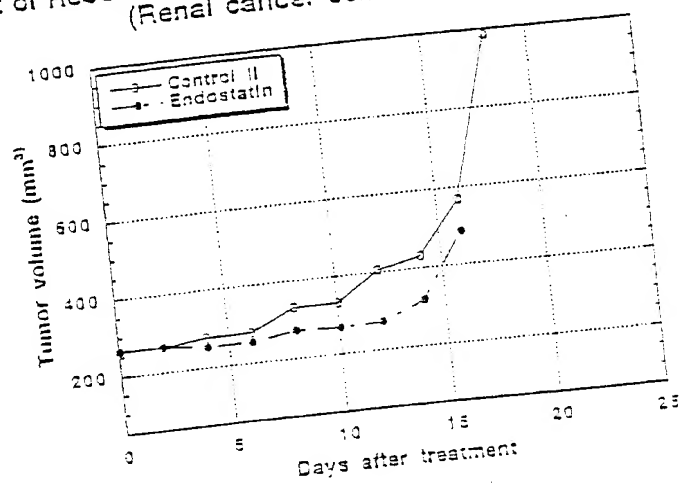
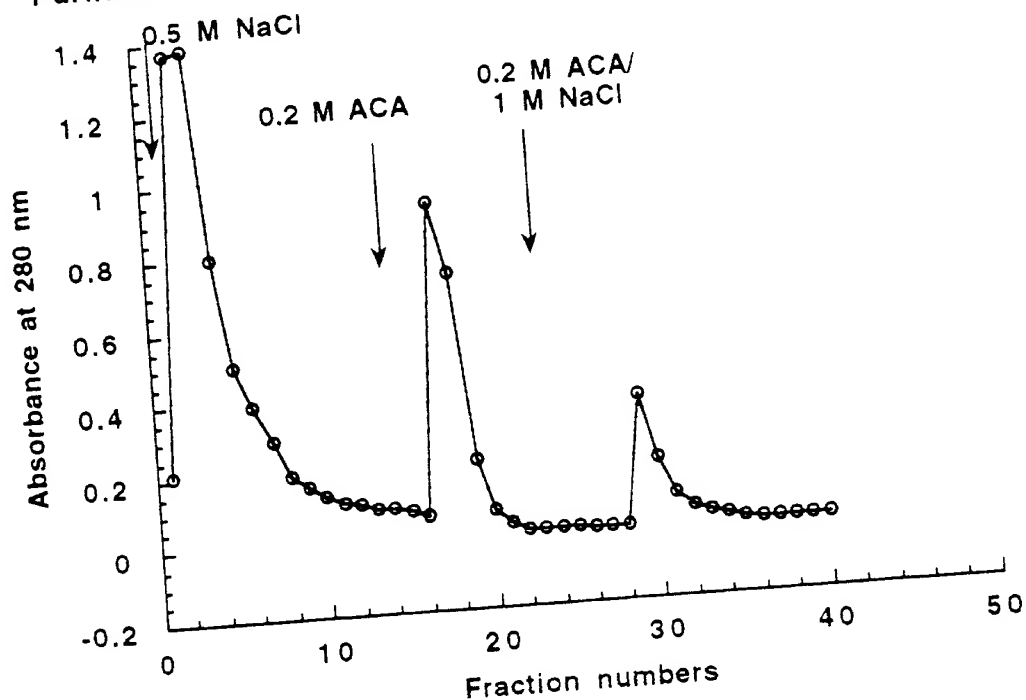


Fig. 20

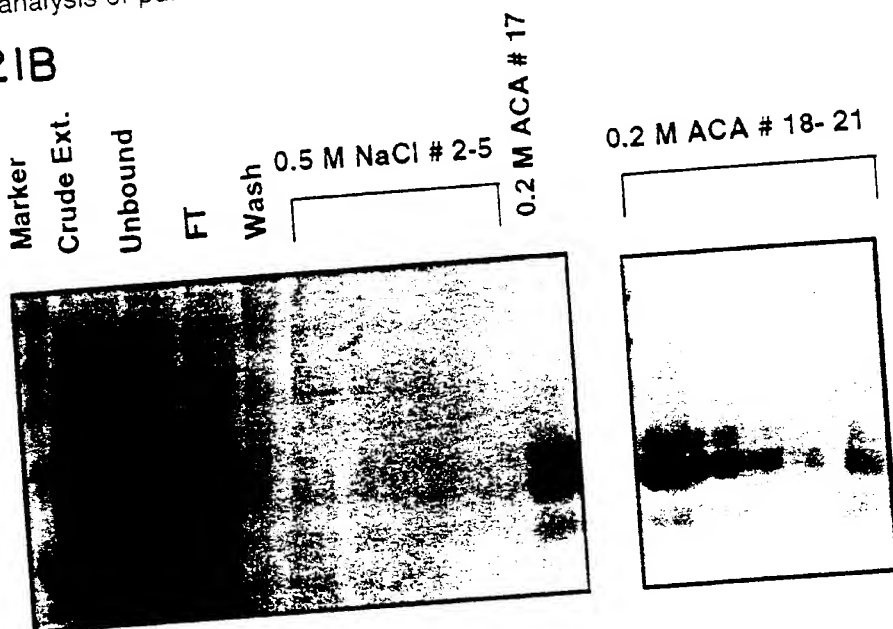
**FIG.2IA**

Purification of Angiostatin (Human):Lysine Sepharose column.



SDS-PAGE analysis of purified recombinant Angiostatin(Human): Lysine Sepharose column

**FIG.2IB**



Sequence Range: 1-546  
 Upstream primers for Restin and Apomigren are underlined,  
 downstream primer for both is double underlined. Primer  
 nucleotides not in the Restin sequence are shown in lower case.

```

    ttt ttt gaa ttc->
      5      10      15      20      25      30      35      40      45
->ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TTG GCT GCT CTG
    TAA AGT TCA CGG TTA ATA CTC TTC GGA CGA GAC GTA AAC CGA CGA GAC
      50      55      60      65      70      75      80      85      90      95
    AAC ATG CCA TTT TCT GGG GAC ATT CGA GCT GAT TTT CAG TGC TTC AAG
    TTG TAC GGT AAA AGA CCC CTG TAA GCT CGA CTA AAA GTC ACG AAG TTC
      100     105     110     115     120     125     130     135     140
    CAG GCC ASA GCT GCA GGA CTG TTG TCC ACC TAC CGA GCA TTC TTA TCT
    GTC CGG TCT CGA CGT CCT GAC AAC AGG TGG ATG GCT CGT AAG AAT AGA
      145     150     155     160     165     170     175     180     185     190
    TCC CAT TTG CAA GAT CTG TCC ACC ATT GTG AGG AAA GCA GAG AGA TAC
    AGG GTA AAC GTT CTA GAC AGG TGG TAA CAC TCC TTT CGT CTC TCT ATG
      195     200     205     210     215     220     225     230     235     240
    AGC CTT CCC ATA GTG AAC CTC AAG GGC CAA GTA CTT TTT AAT AAT TGG
    TCG GAA GGG TAT CAC TTG GAG TTC CCG GTT CAT GAA AAA TTA TTA ACC
      245     250     255     260     265     270     275     280     285
    GAC TCA ATT TTT TCT GGC CAC GGA GGT CAG TTC AAT ATG CAT ATT CCA
    CTG AGT TAA AAA AGA CCG GTG CCT CCA GTC AAG TTA TAC GTA TAA GGT
      290     295     300     305     310     315     320     325     330     335
    ttc cat atg->
->ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA GAT CCT TCT TGG CCC
    TAT ATG AGG AAA CTA CCA GCT CTG TAT TAC TGT CTA GGA AGA ACC GGG
      340     345     350     355     360     365     370     375     380
    CAG AAA GTC ATT TGG CAT GGC TCC AGC CCC CAT GGC GTC CGC CTT GTG
    GTC TTT CAG TAA ACC GTA CCG AGG TCG GGG GTA CCG CAG GCG GAA CAC
      385     390     395     400     405     410     415     420     425     430
    GAT AAC TAC TGT GAA GCA TGG CGA ACC GCG GAC ACA GCG GTC ACG GGA
    CTA TTG ATG ACA CTT CGT ACC GCT TGG CGC CTG TGT CGC CAG TGC CCT
      435     440     445     450     455     460     465     470     475     480
    CTT GGC TCC CCG CTG AGC ACG GGG AAG ATT CTG GAC CAG AAA GCA TAC
    GAA CCG AGG GGC GAC TCG TGC CCC TTC TAA GAC CTG GTC TTT CGT ATG
      485     490     495     500     505     510     515     520     525
    AGC TGT GCT AAT CGG CTA ATT GTC CTA TGT ATC GAA AAC AGT TTC ATG
    TCG ACA CGA TTA CCC GAT TAA CAG GAT ACA TAG CTT TTT TCA AAG TAC
      530     535     540     545
    ACA GAC GCT AGG AAG TAA
    TGT CTG CGA TCC TTC ATT CCG CCG CGT AAG AA
  
```

Figure 22



Flow Chart: Cloning of Restin (ColXV) into Pichia Expression System

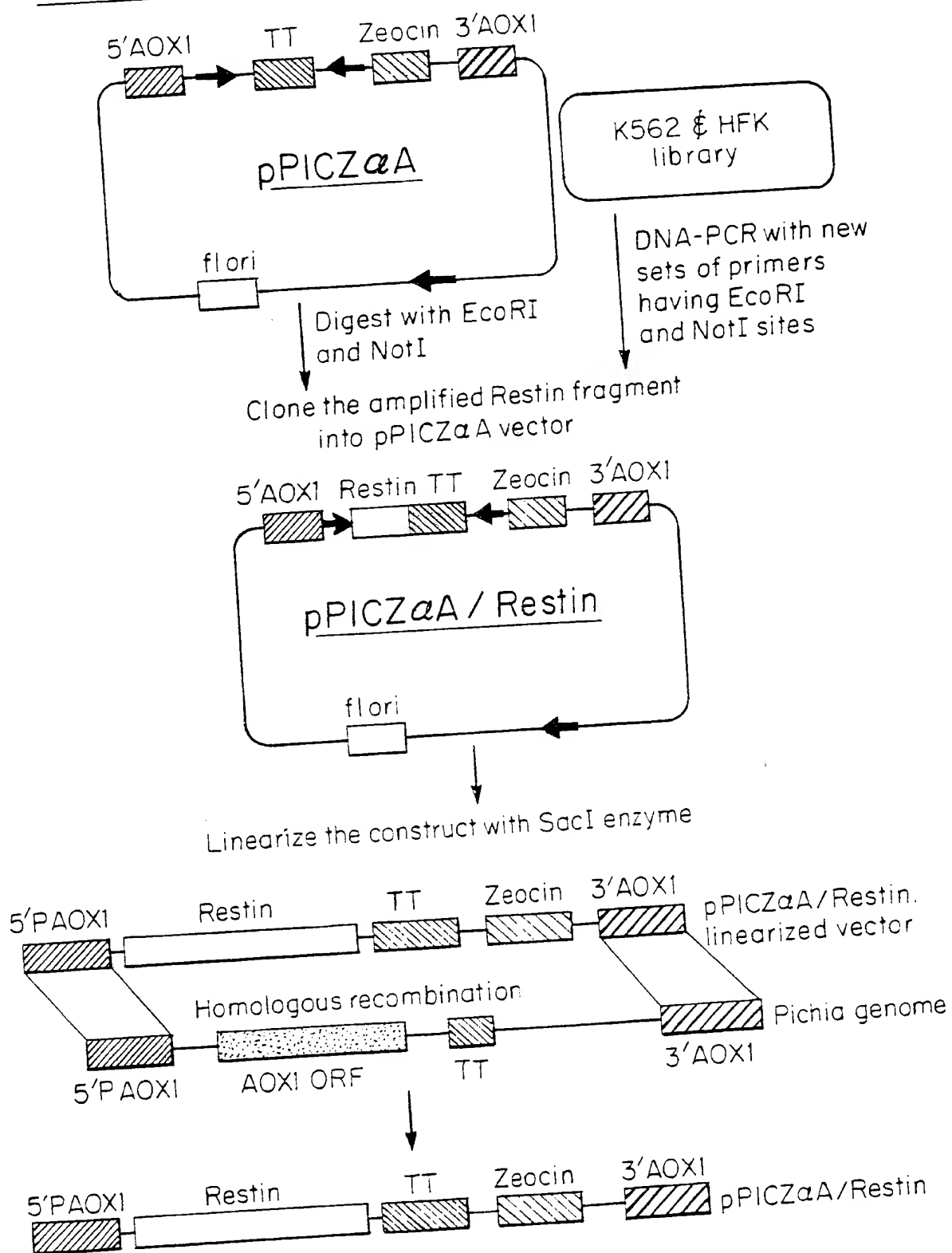


Fig. 24

Sequence Range: 1 to 131

5 10 15 20 25 30 35 40 45  
ISS ANY EKP ALH LAA LNM PFS GDI RAD FQC FKQ ARA AGL LST YRA FLS  
50 55 60 65 70 75 80 85 90 95  
SHL QDL STI VRK AER YSL PIV NLK GQV LFN NWD SIF SGH GGQ FNM HIP  
100 105 110 115 120 125 130 135 140  
IYS FDG RDI MTD PSW PQK VIW HGS SPH GVR LVD NYC EAW RTA DTA VTG  
145 150 155 160 165 170 175 180  
LAS PLS TGK ILD QKA YSC ANR LIV LCI ENS FMT DAR K

# HUMAN RESTIN

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ Restin	5' TTT TTT GAA TTC ATT TCA AGT- GCC AAT TAT GAG AAG CCT GCT- CTG CAT- TTG-3' (up) (SEQ ID NO:21)	<i>Eco</i> RI & <i>Nde</i> I	Eukaryotic (Yeast), Pichia, pPICZαA ( <i>yeast human restin</i> )	EF-restin (SEQ ID NO:27)
	5' AAG AAT GCG GCC GCT TAC TTC- CTA GCG TCT GTC ATG AAA CTG- TTT TCG AT-3' (down) (SEQ ID NO:22)			
pPICZαA/ His.Restin	5' AAT TCC ATC ACC ATC ACC ATC- ACG- 3' (up) (SEQ ID NO:23)	<i>Eco</i> RI (oligo insertion)	Eukaryotic (Yeast), Pichia, pPICZαA ( <i>yeast human his.restin</i> )	EFHHHHHHH-restin (SEQ ID NO:28)
	5' AAT TCG TGA TGG TGA TGG TGA- TGG-3' (down) (SEQ ID NO:24)			
pET28a/ apomigren	5' TTC CAT ATG ATA TAC TCC TTT- GAT GGT CGA GAC ATA ATG ACA-3' (up) (SEQ ID NO:25)	<i>Nde</i> I & <i>Nde</i> I	Prokaryotic, pET system ( <i>E. coli human apomigren</i> )	MGSSHHHHHHSSGLVPRGSHM-apo migren (SEQ ID NO:29)
	5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)			
pPICZαA/ apomigren	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11)	<i>Eco</i> RI & <i>Nde</i> I	Eukaryotic (Yeast), Pichia, pPICZαA ( <i>yeast human apomigren</i> )	EFMGSSHHHHHHSSGLVPRGSHM- apomigren (SEQ ID NO:30)
	5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)			

Fig. 25

No. of cells migrated

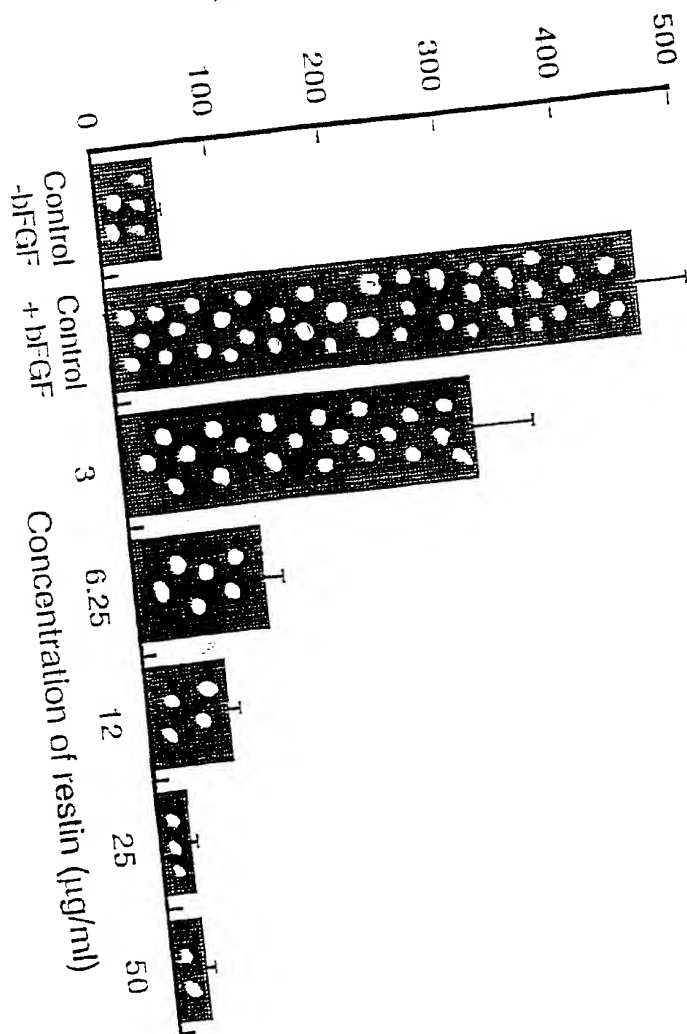


Fig. 27

